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A biotin-streptavidin signal amplification strategy for highly sensitive chemiluminescent immunoassay of chicken interferon-γ

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Experimental

Materials and reagents: The purified recombinant ChIFN-γ protein from E Coli (0.33 mg mL^{-1}), purified monoclonal ChIFN-γ antibody (3.34 mg mL^{-1}), biotinylated monoclonal ChIFN-γ antibody (0.76 mg mL^{-1}, dilution of 1:1000 ratio in use), and horseradish peroxidase (HRP) labeled streptavidin (1:1000 dilution in use) were made by Jiangsu Key Lab of Zoonosis of Yangzhou University. Carboxyl group activated paramagnetic microspheres (PMs) in an aqueous suspension with the mean diameter of 1.5 μm and concentration of 20 mg mL^{-1} were bought from Bangs Laboratories Inc. *N*-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO), respectively. 2-Morpholinoethanesulfonic acid and hydrogen peroxide (30%, H_2O_2) was from Sinopharm Chemical Reagent Co. Ltd (China). *p*-Iodophenol (PIP) was from Alfa Aesar China Ltd and luminol (Acros, Belgium) stock solution (0.01 M) was prepared in 100 mL of 0.1 M NaOH. Prior to use luminol and PIP stock solutions were mixed and diluted using 0.1 M pH 8.5 Tris-HCl buffer. The HRP substrate solution consists of luminol (5 mM)-PIP (0.6 mM)-H_2O_2 (4 mM). Activation buffer for carboxyl group was 0.01 M 2-morpholinoethanesulfonic acid, adjusted to pH 5.5 with NaOH. Coupling buffer for ChIFN-γ antibody immobilization was 0.01 M

pH 7.4 phosphate buffer solution (PBS). Blocking buffer was 0.01 M pH 7.4 PBS containing 1% BSA. To minimize unspecific adsorption, 0.05% Tween-20 was spiked into PBS as wash buffer (PBST). All other reagents were of the best grade available and used as received.

Apparatus: The constructed flow-through CL immunoassay system for ChIFN-γ was illustrated in Scheme 1. Flow-through CL measurements were performed with an IFFM-E Luminescent Analyzer (Remex Analytical Instrument Co., Ltd., Xi'an China). The Teflon tubes (0.8 mm i.d.) and silicon rubber tubes (1.0 mm i.d.) were used to connect all components in this system. All fluids were delivered with a multichannel bidirectional peristaltic pump. The introduction of different solutions into the one-way channel was performed using a multiposition valve with five inlets and one outlet. A glass tube of about 30 μL (i.d. 1.0 mm, length 3.9 cm) used for PMs capture, was positioned in front of photomultiplier (PMT). CL signals produced in glass tube were measured with the PTM operated at -800 V. Instrument control and data record were performed using IFFM software package run under Windows 2003.

Preparation of natural ChIFN-γ: Spleen of 8-week-old specific pathogen free chickens (provided by comparative medical center of Yangzhou University) was moved away sterilely, and single cell suspension was prepared according to previous method.[S1] Splenocytes were adjusted to 10^7 cells mL^{-1} in RPMI1640 (GIBCO) containing 10% (v/v) fetal bovine serum (Hyclone) and 5% (v/v) penicillin-streptomycin solution (Sino-American Biotechnology Company). 250 μL cells per well were transferred into flat-bottomed 24-well plates. Equal volumes of medium with 10% of inactivated fetal bovine serum containing Con A (24 μg mL^{-1}, Sigma) were added in triplicate. The cells were incubated at 4 ^{o}C in 5% CO_2, and cultures were incubated for 4 days. Negative controls received 250 μL RPMI1640 medium only. After 96 h of incubation, the supernatant were harvested from each well for the measurement of ChIFN-γ production.

Preparation of anti-ChIFN-γ antibody immobilized PMs: 125 μL of PMs suspension was washed for three times with activation buffer and separated with a rare earth magnet to remove the supernatant waste. The obtained PMs were resuspended in 500 μL of activation buffer, in which 13 mg of EDC and 8 mg of

NHS were added to activate the carboxyl groups for 1 h under constant stirring at room temperature (RT). The activated PMs were washed three times with coupling buffer and resuspended 500 μL of coupling buffer. 500 μL of 200 μg mL^{-1} anti-ChIFN-γ solution was added and reacted with activated PMs under gentle stir for 2 h at RT followed with standing overnight at 4 ^{o}C. The resulting ChIFN-γ antibody immobilized PMs were thoroughly washed with washing buffer, blocked with blocking buffer for 12 h at 4 ^{o}C, and dispersed in pH 7.4 PBS for storage at 4 ^{o}C.

Immunoassay protocol: The flow-through CL assay process for ChIFN-γ was illustrated in Scheme 1 and given in Table S1. A mixture of ChIFN-γ sample, anti-ChIFN-γ immobilized PMs and 0.76 μg mL^{-1} of biotinylated ChIFN-γ antibody (10 μL for each) was firstly introduced into the glass tube at a flow rate of 1.0 mL min^{-1} and incubated under stop flow at RT for 20 min. PBST was then introduced at a flow rate of 1.0 mL min^{-1} into the system to wash the formed immunocomplex immobilized PMs for 2 min with a magnet. Next, 30 μL of HRP labeled streptavidin was delivered into the glass tube and incubated under stop flow at RT for 15 min. Finally, 30 μL of CL substrate solution was introduced into the glass tube. When the HRP catalyzed CL reaction was triggered under stop flow, the magnetic field was withdrawn and CL signals were collected. The whole procedure from sample injection to signal collection could be finished within 47 min. At a time of about 48 min, the flow-through CL immunoassay system could be ready for next assay cycle.

Table S1 Details of the proposed flow-through CL immunoassay for the determination of ChIFN-γ

Step no.	Valve position	Step	Starting time (min: s)
1	1	Introduce the mixture of 10 μL sample, 10 μL ChIFN-γ antibody immobilized PMs, 10 μL biotinylated ChIFN-γ antibody into the glass tube	00:00
2	1	Stop flow and incubate at RT	00:30
3	2	Wash the formed immunocomplex immobilized PMs in glass tube with PBST at a flow rate of 1.0 mL min^{-1} by a magnet	20:30
4	1	Introduce 30 μL HRP labeled streptavidin into the glass tube	22:30
5	1	Stop flow and incubate at RT	23:00
6	2	Wash the glass tube with PBST at a flow rate of 1.0 mL min^{-1} by a magnet	38:00
7	3	Introduce 30 μL HRP substrate into the glass tube, stop flow and withdraw the magnetic field to collect CL signal	41:00
8	2	Wash the glass tube with PBST at a flow rate of 1.0 mL min^{-1}	47:00
9	1	ready for the next assay cycle	48:00

References

S1 C. H. Yun, H. S. Lillehoj and K. D. Choi, *Vet. Immunol. Immunopathol.*, 2000, **73**, 297-308.